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CONCERNING A FILING UNDER 35 U.S.C. 371

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09/856275

International Application. No.

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Priority Date Claimed

PCT/FR99/02827

November 18, 1999

November 18, 1998

Title of Invention

GENE CODING FOR AN ACYLTRANSFERASE OF OIL SEED RAPE AND USES THEREOF

Applicants For DO/EO/US

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Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. [X] has been transmitted by the International Bureau.
 - c. [] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
 - a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. [] have been transmitted by the International Bureau.
 - c. [] have not been made; however, the time limit for making such amendments has NOT expired.
 - d. [X] have not been made and will not be made.
8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [] An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. [] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
[] A SECOND or SUBSEQUENT preliminary amendment.
14. [X] Other items or information:
 - a. [X] WO 00/29585 - (front page)
 - b. [X] PCT/IB/304
 - [X] PCT/IB/308
 - [X] PCT/IPEA/409
 - [X] Paper Copy of Sequence Listing
 - [X] Diskette with Sequence Listing in C.R.F.
 - [X] Statement Accompanying Sequence Listings

U.S. APPLICATION NO. **09/856275** INTERNATIONAL APPLICATION NO. _____ ATTORNEY DOCKET NUMBER _____
 Unpublished PCT/FR99/02827 045636-5046

15. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....\$860.00

International preliminary examination fee paid to

USPTO (37 CFR 1.482).....\$690.00

No international preliminary examination fee paid to

USPTO (37 CFR 1.482) but international search fee

paid to USPTO (37 CFR 1.445(a)(2)).....\$710.00

Neither international preliminary examination fee

(37 CFR 1.482) nor international search fee

(37 CFR 1.445(a)(2)) paid to USPTO.....\$1000.00

International preliminary examination fee paid to USPTO

(37 CFR 1.482) and all claims satisfied provisions

of PCT Article 33(2)-(4).....\$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = \$ 860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than

[] 20 [] 30 months from the earliest claimed priority date

(37 CFR 1.492(e)).

\$

Claims	Number Filed	Number Extra	Rate	
Total Claims	40 - 20 =	20	X \$18.00	\$ 360.00
Independent Claims	1 - 3 =	0	X \$80.00	\$
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$ 270.00

TOTAL OF ABOVE CALCULATIONS = \$

Reduction by 1/2 for filing by small entity, if applicable. Verified

Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)

-\$

SUBTOTAL = \$1490.00

Processing fee of \$130.00 for furnishing the English translation later

than [] 20 [] 30 months from the earliest claimed priority date

(37 CFR 1.492(f)).

+\$

TOTAL NATIONAL FEE = \$1490.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The

assignment must be accompanied by an appropriate cover sheet

(37 CFR 3.28, 3.31).

\$40.00 per property

+\$

TOTAL FEES ENCLOSED = \$ 1490.00

Amount to be

refunded

\$

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\$

a. [] A check in the amount of \$-0- to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 50-0310 for \$1490.00

to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] **Except** for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 CFR §1.16 and §1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

Customer No. 009629

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PATENT
Attorney Docket 045636-5046

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Michel RENARD *et al.***)
)
National Stage of)
International Application: **PCT/FR99/02827**)
)
International Filing Date: **November 18, 1999**)
)
Application No. **Not Assigned**) Group Art Unit:
) **Not Assigned**
Date of National Stage Entry: **May 18, 2001**)
) Examiner:
) **Not Assigned**
For: **GENE CODING FOR AN**)
 ACYLTRANSFERASE OF OIL SEED)
 RAPE AND USES THEREOF)
)

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application, please amend the application as follows:

In the Claims:

Please cancel claims 1 - 12 without prejudice or disclaimer.

Please add claims 13 - 25 as follows:

13. A nucleic acid fragment comprising a sequence encoding a plant lysophosphatidic acid acyltransferase (LPAAT), the peptide sequence of which exhibits at least 20% identity with the peptide sequence SEQ ID NO: 2.

14. A nucleic acid fragment comprising a sequence that is complementary to the coding sequence of claim 13.

15. The nucleic acid fragment of claim 13, wherein said coding sequence encodes the polypeptide of sequence SEQ ID NO: 2.

16. A nucleic acid fragment comprising at least 20 base pairs, which is capable of hybridizing specifically, under stringent conditions, with a sequence as defined in any one of claims 13, with the exception of the fragments consisting of an oligonucleotide encoding one of the following peptide sequences:

FPEGTRS;

PFKKGA;

or of the sequence complementary thereto.

17. A recombinant vector containing a nucleic acid fragment as claimed in any one of claims 13 -16.

18. A cell transformed with at least one nucleic acid fragment as claimed in any one of claims 13 -16.

19. The transformed cell as claimed in claim 18, wherein the cell is a plant cell.
20. A transgenic plant transformed with at least one nucleic acid fragment as claimed in any one of claims 13 -16.
21. A method of regulating the LPAAT activity of a plant comprising transforming a plant with at least one nucleic acid fragment as claimed in any one of claims 13 -16.
22. The method of claim 21 wherein the plant is rapeseed.
23. The method as claimed in either claim 21 wherein said nucleic acid is present in the cells of the transformed plant in an antisense orientation.
24. The method as claimed in either claim 21 wherein said nucleic acid is present in the transformed cell in a sense orientation and the transformed plant expresses functional LPAAT.
25. The method of claim 24 wherein said functional LPAAT does not include a signal peptide capable of importing the LPAAT into the membrane of the chloroplasts.

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REMARKS

Applicants respectfully submit that no prohibited new matter has been introduced by this Preliminary Amendment and that claims 13 to 25 are drawn to the same invention as claims 1-12 of International Application PCT/FR99/02827. The changes to the claims represent changes in formalities so as to bring the claims into compliance with the rules of practice in the United States, such as: "use" claims are not a recognized category of invention (see original claims 8-12); to provide established claim terminology to describe the intended scope of the claims, i.e. incorporation of the terms "comprising" and "wherein" rather than "containing" and "characterized in that" (see claims 2, 4 and 6); to avoid optional language such as "and/or" (original claim 1) which is not common practice for claims in U.S. patent applications and to correct grammar such as noun placement and tense (see all of the original claims). These changes do not narrow the claimed subject matter presented and examined in the corresponding International Application.

Respectfully submitted,

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Date: **May 18, 2001**
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GENE CODING FOR AN ACYLTRANSFERASE OF OIL SEED RAPE,
AND USES THEREOF

The invention relates to the identification and
5 to the cloning of a gene encoding an acyltransferase,
and to uses thereof.

In plants, glycerolipids (glycolipids,
phospholipids and triacylglycerides) constitute the
major portion of lipids. Their common precursor is *sn*-
10 1,2-diacylglycerol-3-phosphate, or phosphatidic acid
(PA), resulting from the esterification of the *sn*-1 and
sn-2 positions of glycerol-3-phosphate (G3P) with fatty
acids. *SN*-glycerol-3-phosphate acyltransferase (GPAT)
(E.C. 2.3.1.15) catalyzes the acylation of the *sn*-1
15 position of G3P so as to form *sn*-1-acylglycerol-3-
phosphate, or lysophosphatidic acid (LPA). The LPA is
then used as a substrate by 1-acyl-*sn*-glycerol-3-
phosphate acyltransferase, or lysophosphatidic
acyltransferase (LPAAT) (E.C.2.3.1.51), which acylates
20 the *sn*-2 position of glycerol. In the synthesis of
triacylglycerides, which constitute most of the storage
lipids, a third enzyme, *sn*-1,2-diacylglycerol
acyltransferase, or diacylglycerol acyltransferase
(DAGAT), is involved, which catalyzes the acylation of
25 the *sn*-3 position.

Plant lipids are currently used in very varied
fields, from foods to the chemical industry, and it is
desirable to have plants which produce lipids
specifically suited to the use envisaged. With this
30 aim, attempts are being made in particular to modify
the fatty acid composition of the glycerolipids, and in
particular of the triacylglycerides.

For example, in the case of rapeseed (*Brassica
napus*), plants having the lowest possible erucic acid
35 content are used for edible oil. On the other hand, a
high erucic acid content is sought in plants which
produce oils intended for industrial use.

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The fatty acid composition of the glycerolipids depends essentially, firstly, on the quantitative and qualitative distribution of the fatty acids produced by the plant and, secondly, on the substrate specificity of the acyltransferases with respect to these fatty acids. In order to control this composition, it has been proposed to act separately or jointly on these two factors, by intervening:

- at the level of biosynthesis of the fatty acids, in order to promote, or on the contrary inhibit, the production of one or more specific fatty acids and, optionally, to induce the synthesis of new fatty acids;
- at the level of acylation of the G3P, in order to modify its specificity in the desired way.

In the case of rapeseed, the most erucic acid-rich varieties currently available produce an oil in which erucic acid represents at most 50 to 60% of the total fatty acids. Analysis of the triacylglycerides of the seeds derived from these varieties has shown that this acid is present virtually exclusively at the *sn*-1 and *sn*-3 positions; this selective distribution has been attributed to the substrate specificity of rapeseed LPAAT, which excludes very-long-chain (>C20) fatty acids; this limits the erucic acid content of the triacylglycerides of seeds from rapeseedseed to a theoretical maximum threshold of 66% of the total fatty acids.

With the aim of avoiding this limitation, the gene of a *Limnanthes alba* LPAAT capable of incorporating erucic acid at the *sn*-2 position has been expressed in the seeds of a variety of rapeseed having a high erucic acid content. However, although incorporation of erucic acid at the *sn*-2 position was effectively observed in the triacylglycerides of the seeds of this transgenic rapeseed, this incorporation remained low; in addition, the total amount of erucic acid incorporated into these triacylglycerides was no greater than that of the nontransformed control plants [LASSNER et al., Plant Physiol. 109:1389-1394, (1995)].

This result may be due, besides the possibility of a limiting production of erucic acid, to poor specificity of the exogenous *Limnanthes* LPPAT which might, besides erucic acid, also incorporate oleic acid, and also to the existence of competition between the exogenous LPAAT activity and endogenous LPAAT activity of the rapeseed, which it would be necessary to inhibit in order to increase the incorporation of erucic acid.

To date, only a small amount of information has been available concerning the enzyme(s) responsible for LPAAT activity in rapeseed. LPAATs are in fact membrane-bound enzymes which are difficult to purify in active form.

With the exception of coconut LPAAT [KNUTZON et al., Plant Physiol. 109:999-1006, (1995)], which has been purified from the membranes of the albumen, and the gene of which has subsequently been isolated by screening a cDNA library, the plant LPAATs already identified have mostly been characterized using techniques of molecular genetics. They are maize LPAAT [BROWN et al., Plant Mol. Biol., 26:211-223, (1994)], and *Limnanthes* LPAATs [BROWN et al., Plant Mol. Biol., 29:267-278, (1995); HANKE et al., Eur. J. Biochem. 232:806-810, (1995)].

In order to enable the control of the acylation at the sn-2 position to be improved, the inventors have undertaken to characterize the enzyme(s) involved in LPAAT activity in rapeseed.

They have thus succeeded in isolating a DNA sequence from *Brassica napus* encoding a functional plastidial LPAAT; this LPAAT will hereinafter be named BAT2 (Brassica AcylTransferase 2).

A nucleic acid sequence comprising the sequence encoding BAT2 is represented in the attached sequence listing under the number SEQ ID NO: 1, and the deduced polypeptide sequence is represented under the number SEQ ID NO: 2.

2 ATG codons which may constitute potential translation initiation sites are present on the sequence SEQ ID NO: 1; the polypeptide of 344 amino acids starting at the methionine residue at position 16 of the sequence SEQ ID NO: 2 is sufficient for the LPAAT activity.

The analysis of the amino acid sequence of BAT2 using the pSORT program suggests the presence of a signal sequence included in the 95 N-terminal amino acids of the sequence SEQ ID NO: 2. This signal sequence is involved in directing the BAT2 LPPAT into the plastidial membrane.

The sequence of the active mature protein is included in the 279 C-terminal amino acids.

The comparison, using the BLASTX2 program [GISH et al., Nat. Genet., 31:266-272, (1994)], between the BAT2 peptide sequence and the LPAAT peptide sequences previously known reveals very weak homology (a maximum of 20% identity) when the comparison is carried out over the entire sequence.

Over certain regions of the sequence, greater homology is observed. Figure 1 represents the alignment of the sequence 187-302 of BAT2 with the sequences of the LPAATs having the strongest homology. The most significant scores are observed with:

- the product of the *S. cerevisiae* *SLC1* gene (P33333) [NAGIAC et al. J. Biol. Chem., 268:22145-22163, (1993)] : 32% identity and 51% equivalence, over an alignment of 204 amino acids;
- the microsomal LPAAT of the seeds from *Limnanthes* (Q42870) [HANKE et al., Eur. J. Biochem., 232:806-810, (1995); LASSNER et al., Plant Physiol. 109:1389-1394, (1995); BROWN et al., Plant Mol. Biol., 29:267-278, (1995)] : 30% identity and 54% equivalence, over an alignment of 182 amino acids;
- the coconut endosperm LPAAT (Q42670) [KNUTZON et al., Plant Physiol. 109:999-1006, (1995)] : 31% identity and 47% equivalence, over an alignment of 229 amino acids;

- the hypothetical *Synechocystis* LPAAT (P74498)
: 30% identity and 55% equivalence, over an alignment
of 143 amino acids:

- the *E. coli* plsC protein (P26647) : 31%
5 identity and 50% equivalence, over an alignment of 115
amino acids.

A subject of the present invention is a nucleic acid fragment comprising:

10 a), a sequence encoding a plant LPAAT, the peptide sequence of which shows at least 20%, preferably at least 30%, and advantageously at least 50 to 95%, identity with the sequence SEQ ID NO: 2; and/or b) a sequence complementary to the coding sequence a) above.

15 According to a preferred embodiment of the present invention, said coding sequence encodes the polypeptide of sequence SEQ ID NO: 2.

The invention also encompasses fragments of more than 20 bp, and preferably of more than 30 bp, which are fragments of a coding sequence as defined above, or which are capable of hybridizing specifically, under stringent conditions, with said sequence. This includes in particular the fragments of any sequence encoding the polypeptide SEQ ID NO: 2, or of the sequence complementary thereto, with the exception of the fragments consisting of an oligonucleotide encoding one of the following peptide sequences (1-letter code):

30 FPEGTRS;
PFKKGA;

which are common to LPAATs having previously known sequences, or of the fragments complementary to said oligonucleotide.

Nucleic acid fragments in accordance with the
35 invention can in particular be used as primers and/or
probes, to detect and clone sequences encoding
plastidial LPAATs, from rapeseed or from other plants,
and also sequences encoding LPAATs from rapeseed or
from other species, in particular from crucifers, which

are expressed in cellular compartments other than
plasts, in particular the endoplasmic reticulum.

The analyses carried out by Southern transfer
and by RFLP, using the BAT2 cDNA, labeled with ^{32}P , as a
5 hybridization probe, under stringent conditions, show
the presence, in the rapeseed genome and also in the *A.*
thaliana genome, of at least 2 homologous copies of the
BAT2 gene, and imply that this gene is part of a
multigene family comprising 4 members.

10 A subject of the present invention is also:

- the recombinant vectors resulting from the
insertion of at least one nucleic acid fragment in
accordance with the invention into a suitable vector;
advantageously, they are expression vectors in which
15 the nucleic acid fragment in accordance with the
invention is inserted under the transcriptional control
of regulatory sequences (such as a promoter and/or
terminator) which are functional in a host cell in
which the expression of said fragment is desired.

20 - the host cells, which may be prokaryotic or
eukaryotic, and the multicellular organisms, in
particular plant cells and plants, transformed with at
least one nucleic acid fragment in accordance with the
invention.

25 The invention also encompasses the recombinant
LPAAT, or the fragments of recombinant LPAAT, resulting
from the expression, in a host cell, of the sequence
encoding said LPAAT or said fragment, which sequence is
carried by a nucleic acid fragment in accordance with
30 the invention. The recombinant LPAAT in accordance with
the invention, or the fragments thereof, can, for
example, be used to produce anti-LPAAT antibodies which
enable cDNA expression libraries to be screened in the
context of detecting and cloning other LPAATs.

35 Nucleic acid fragments in accordance with the
invention can advantageously be used, in the sense or
antisense orientation, to produce transgenic plants, in
particular from rapeseed or from other oil-producing
plants, in order to regulate the LPAAT activity in the

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plant thus transformed, and to act on the fatty acid composition of the lipids, and in particular of the triacylglycerides, produced by this plant.

The present invention also encompasses the
5 transgenic plants produced in this way.

These plants can be produced using the conventional techniques, which are known in themselves, of plant transgenesis. Depending on the use envisaged, a nucleic acid sequence in accordance with the invention can be placed under the control of an inducible promoter or of a constitutive promoter, of a ubiquitous promoter or of a tissue-specific promoter. These plants can also contain other transgenes, preferably transgenes derived from genes involved in lipid biosynthesis.

It is possible in particular to produce:

- transgenic plants expressing at least one sequence in accordance with the invention encoding a functional LPAAT, instead of and in place of one or
20 more sequences encoding endogenous LPAATs, or in addition to these sequences;

- transgenic plants expressing at least one sequence in accordance with the invention in antisense orientation, in order to inhibit the expression of the homologous endogenous LPAATs and thus promote the activity of other LPAATs, of endogenous or exogenous origin.

For example:

- in order to produce transgenic rapeseed plants having a high erucic acid content, the rapeseed plant can be cotransformed with, firstly, a DNA sequence encoding an LPAAT which preferentially incorporates the erucic acid at the sn-2 position, such as the *Limnanthes alba* LPAAT [LASSNER et al., (1995), 30 abovementioned publication], and, secondly, a nucleic acid sequence in accordance with the invention in antisense orientation, in order to inhibit, at least partially, the production of endogenous LPAAT which

competes with the activity of the exogenous *Limnanthes* LPAAT;

5 - in order to increase the overall triglyceride content of the seeds, the rapeseed plant can be transformed with a DNA sequence in accordance with the invention which encodes a plastidial LPAAT deleted of its sequence for being directed into plasts;

10 - in order to increase the saturated fatty acid content, in particular the palmitic acid content, the rapeseed plant can be cotransformed with a DNA sequence in accordance with the invention which encodes a plastidial LPAAT deleted of its sequence for being directed into chloroplasts, and with one or more genes for ACP-thioesterases which preferentially use
15 palmitoyl-ACPs as substrates.

The present invention will be more clearly understood with the aid of the further description which follows, which refers to nonlimiting examples illustrating the identification and cloning of a gene
20 encoding the *Brassica napus* BAT2 LPAAT.

EXAMPLE 1: ISOLATION AND CHARACTERIZATION OF THE cDNA OF A RAPESEED LPAAT

In order to search for the presence of genes encoding LPAATs, a rapeseed immature embryo cDNA
25 library was screened by heterologous complementation of the mutation of the *plsC* gene of the *E. coli* strain JC201 [COLEMAN, J. Biol. Chem., 265:17215-17221, (1990)]. This point mutation confers a heat-sensitive phenotype on the JC201 mutants, due to the inactivation
30 at high temperature of the LPAAT encoded by the *plsC* gene. These mutants grow well at 30°C, with difficulty at 37°C, and not at all at 42-44°C.

For the screening, a phagemid library derived from 2×10^6 clones taken from a rapeseed immature
35 embryo cDNA library and cloned into the vector lambda ZAPII (STRATAGENE), was constructed using the "ExAssist" kit (STRATAGENE).

The bacteria are transformed with these phagemids by electroporation, and then cultured on LB

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agar, in the presence of ampicillin and IPTG (isopropyl- β -D-galactothioipyranoside). The bacteria which grow at 42°C are selected. The plasmid DNA of the clones capable of growing at 42°C was analyzed by PCR in order to determine the size of the insert. After 3 cycles of transformation followed by selection, approximately 85% of the clones contain an insert of approximately 1.2 kb. The sequencing of the ends of the inserts of 4 of these clones shows that they are identical. One of these clones, named pBAT2, was entirely sequenced.

EXAMPLE 2: NUCLEOTIDE SEQUENCE OF BAT2 AND DEDUCED PEPTIDE SEQUENCE.

The cDNA of the pBAT2 clone comprises a 1155 bp sequence followed by an 18 residue poly(A) tail. This sequence comprises a single open reading frame, corresponding to a 351 amino acid polypeptide, which represents a fusion protein, comprising 344 amino acids of the pBAT2 sequence, and a portion of the β -galactosidase sequence from the cloning vector. The sequence which is represented on the attached sequence listing under the number SEQ ID NO: 1 also comprises a portion of the genomic sequence (nucleotides 1 to 79 of the sequence SEQ ID NO: 1) located upstream of the pBAT2 cDNA sequence.

2 ATG codons which may constitute potential translation initiation sites were located on the sequence SEQ ID NO: 1; if the first one of them (position 58 of the sequence SEQ ID NO: 1) is used, the product of translation of the sequence SEQ ID NO: 1 is a 359 amino acid polypeptide, the theoretical molecular weight and pI of which are, respectively, 39.6 kDa and approximately 9.8; this polypeptide is represented on the attached sequence listing under the number SEQ ID NO: 2. If the translation initiation takes place at the 2nd ATG codon (position 103 of the sequence SEQ ID NO: 1), the translation product is a 344 amino acid polypeptide, the theoretical molecular weight of which is approximately 37.9 kDa.

The analysis of the BAT2 amino acid sequence using the pSORT program suggests the presence of a signal peptide of approximately 80 to 95 residues. This potential signal sequence is rich in serine, in alanine, in valine and in basic amino acids, which is characteristic of sequences for directing toward the chloroplast membrane.

The analysis of the polypeptide sequence also indicates the presence of two potential transmembrane domains, located, respectively, between amino acids 124 to 140, and 219 to 235.

The consensus sequences of the LPAATs (FPEGTRS and PFKKGA) are located, respectively, at positions 273-279 and 286-291 of the sequence SEQ ID NO: 2; a sequence corresponding to the consensus sequence NHXXXXD, which is conserved in all the membrane-bound acyltransferases known to date, is located at positions 202-208 of the sequence SEQ ID NO: 2.

EXAMPLE 3: ENZYMATIC ACTIVITY OF THE PROTEIN ENCODED BY THE pBAT2 cDNA INSERT

In order to verify that the protein encoded by the pBAT2 insert effectively had an LPAAT activity, the capacity of this protein to incorporate oleic acid or palmitic acid at the *sn*-2 position of LPA was tested.

The *E. coli* strain JC201 transformed with pBAT2 and, by way of controls, the *E. coli* strain JC201 either nontransformed or transformed with the vector (pBSK) lacking the BAT2 cDNA insert are cultured at 30°C until an optical density of 0.5 is reached.

After induction with IPTG and culturing for 3 h at 30°C, the bacteria are lysed and fractionated and the LPAAT specific activity is measured, on the crude membrane extracts, in the presence of oleoyl-CoA(1-¹⁴C) or of palmitoyl-CoA(1-¹⁴C), according to the protocol of CAO et al. [Plant Physiol., 9:1199-1206, (1990)].

The results are shown in Table I below, which gives the specific activity, in pmol of phosphatidic acid formed/mg of protein/hour.

TABLE I

CULTURE	SUBSTRATE	
	Oleoyl-CoA(1- ¹⁴ C)	Palmitoyl-CoA(1- ¹⁴ C)
JC201	1.86	3.74
JC201+pBSK	1.65	1.59
JC201+pBAT2	5.8	11.06

The membrane extracts of the culture transformed with pBAT2 exhibit an LPAAT activity which is greater than that of the membrane extracts obtained from the nontransformed culture or the culture transformed with the vector pBSK, which shows that the LPAAT activity is effectively restored by the product of translation of the pBAT2 insert.

EXAMPLE 4: CELLULAR LOCATION OF BAT2

The plastid location suggested by the BAT2 sequence analysis was verified by testing the capacity of isolated pea chloroplasts to import BAT2.

With this aim, the cDNA of the pBAT2 clone was transcribed *in vitro*, using T3 RNA polymerase; the transcript is translated in the wheat germ acellular system, in the presence of ³⁵S-labeled methionine. A translation product of approximately 40 kDa is thus obtained. This product is incubated with isolated pea chloroplasts. After incubation, the chloroplasts are treated with protease and fractionated, according to the protocol described by BROCK et al. [Plant Mol. Biol. 23(4), 717, (1993)], and the various fractions are analyzed by electrophoresis in order to search for the ³⁵S-labeled product.

The results of this analysis show that the BAT2 translation product is imported into the pea chloroplasts and cleaved into a 32 kDa protein which is essentially located in the membrane fraction, and an 8 kDa signal peptide.

These results confirm that the BAT2 protein is indeed synthesized with a signal peptide, the role of which is to import the protein into the membrane of the chloroplasts. The precursor has an apparent mass of

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approximately 40 kDa and the signal peptide has an apparent mass of approximately 8 kDa.

EXAMPLE 5 : LOCATION OF THE EXPRESSION OF THE BAT2 GENE

The expression of the *BAT2* gene was studied in various organs of *B. napus* and of *A. thaliana*.

The study was carried out by Northern transfer, using a probe corresponding to the *BAT2* coding sequence, on total RNAs of stems, roots, leaves, flowers, seeds undergoing development [28 DAP (days after pollination)] and dry seeds.

In each of the *B. napus* and *A. thaliana* tissues tested, hybridization of the probe with a transcript of approximately 1.3 kb in *B. napus*, and with a transcript of approximately 1 kb in *A. thaliana*, is observed. The strength of the hybridization signal is similar in all the tissues, including the nonphotosynthetic tissues containing plasts other than chloroplasts. The observation of hybridization in mature seeds indicates, in addition, that the *BAT2* message remains stable during maturation of the seed.

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- 13 -

CLAIMS

- 1) A nucleic acid fragment comprising:
- a) a sequence encoding a plant LPAAT, the peptide sequence of which shows at least 20% identity with the sequence SEQ ID NO: 2; and/or
 - b) a sequence complementary to the coding sequence a) above.
- 2) The nucleic acid fragment as claimed in claim 1, characterized in that said coding sequence encodes the polypeptide of sequence SEQ ID NO: 2.
- 3) A nucleic acid fragment of more than 20 bp, capable of hybridizing specifically, under stringent conditions, with a coding sequence as defined in either of claims 1 and 2, with the exception of the fragments consisting of an oligonucleotide encoding one of the following peptide sequences:
- FPEGTRS;
PFKKGA;
- or of the sequence complementary thereto.
- 4) A recombinant vector containing a nucleic acid fragment as claimed in any one of claims 1 to 3.
- 5) A cell transformed with at least one nucleic acid fragment as claimed in any one of claims 1 to 3.
- 6) The transformed cell as claimed in claim 5, characterized in that it is a plant cell.
- 7) A transgenic plant transformed with at least one nucleic acid fragment as claimed in any one of claims 1 to 3.
- 8) The use of a nucleic acid fragment as claimed in any one of claims 1 to 3, for regulating LPAAT activity in a plant.
- 9) The use as claimed in claim 8, characterized in that said plant is rapeseed.

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10) The use as claimed in either of claims 8 and 9, characterized in that said nucleic acid fragment is used in the antisense orientation.

11) The use as claimed in claim 10,
5 characterized in that said nucleic acid fragment is used to express a functional LPAAT.

12) The use as claimed in claim 11, characterized in that said LPAAT is deleted of its sequence for being directed into plasts.

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEYU.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO. :

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

GENE CODING FOR AN ACYLTRANSFERASE OF OIL SEED RAPE AND USES THEREOF

the specification of which :

is attached hereto ; or

was filed as United States application Serial No. _____ on _____ and was amended on _____ (if applicable) ; or

was filed as a PCT international application Number PCT/FR99/02827 on November 18, 1999 and was amended under PCT article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed :

PRIOR FOREIGN APPLICATION(S) :

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
FRANCE	98/14470	18 November 1998	X

Combined Declaration For Patent Application and Power of Attorney – (Continued)
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO. :

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.

U.S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365 (c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S Patent and Trademark Office all information known to me to be material to the patentability of claims represented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application :

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT :

U.S. APPLICATIONS

STATUS (Check one)

U.S. APPLICATION NO.

U.S. FILING DATE

PATENTED

PENDING

ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct all correspondence be addressed to that Customer Number.


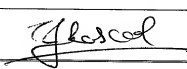

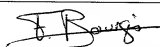
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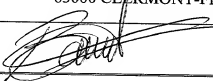

Combined Declaration For Patent Application and Power of Attorney-- (Continued)
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
EIGHTH INVENTOR'S SIGNATURE		DATE
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RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
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NINTH INVENTOR'S SIGNATURE		DATE

SEQUENCE LISTING

<110> INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE
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BARRET, Pierre
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<120> GENE CODING FOR AN ACYLTRANSFERASE OF OIL SEED RAPE,
AND USES THEREOF

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